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Women with germline mutations in the breast and ovarian cancer gene 1 (*Brca1*) have an approximately 50% lifetime of developing ovarian cancer and almost 90% chance of breast cancer. Brca1 mutations account for a significant percentage of all breast cancer cases. It appears that the main role for the Brca1 protein in cells is to prevent the accumulation of mutations in key growth regulatory genes n response to DNA damage. BRCA1 is phosphorylated in response to DNA damage by an elaborate surveillance mechanism called a checkpoint, which detects DNA damage and prevents the accumulation of mutation. We are investigating the role these phosphorylation events play in the regulation of BRCA1. We have mapped phosphorylation sites and will mutate them to determine their function. We are also planning to investigate the mechanism through with the BRCA1 protein localizes to sites of DNA damage within cells.

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Introduction

Maintenance of genomic integrity is crucial for the development and health of organisms. Cell cycle checkpoints and DNA repair mechanisms help ensure the distribution of an intact genome to all cells and progeny. The inactivation of many of the genes involved in these activities have been linked to syndromes that cause a predisposition to cancer in humans. The *ATM*, *Brca1*, and *Brca2* genes are three such tumor suppressors involved in preventing genetic damage (1). Mutations in *ATM* cause ataxia telengiectasia (A-T), a disorder characterized by atrophy of the cerebellum and thymus, immunodeficiency, premature aging, predisposition to cancer, and sensitivity to ionizing radiation (2). Furthermore, heterozygous carriers of a dysfunctional ATM gene are predisposed to breast cancer (3). Mutations in *Brca1* and *Brca2* are linked to inherited, early-onset breast cancer (4). Mutations in *Brca1*, *Brca2*, or *ATM* cause defects in cellular proliferation, genomic instability, and sensitivity to DNA damage (5-7).

ATM is a member of a protein family related to phosphoinositide kinases that includes ATR, *MEC1*, *TEL1* and *RAD3*. These proteins are essential for signaling the presence of DNA damage and activating cell cycle checkpoints (8). ATM is activated in response to DNA damage and is required for efficient DNA double strand break repair and optimal phosphorylation and activation of the p53, c-Abl, and Chk2 proteins that promote apoptosis or cell cycle arrest (9-14).

The Brca1 and Brca2 proteins form a complex with Rad51, a RecA homologue required for homologous recombinational repair of DNA double stranded breaks (6,15-17). These three proteins localize to discrete nuclear foci during S phase of the cell cycle, share developmental expression patterns, and are maximally expressed at the G1-S transition (16-19). Brca1 mutations in mice result in genetic instability, defective G2/M checkpoint control and reduced homologous recombination (7). Exposure of cells to ionizing radiation or hydroxyurea causes dispersal of Brca1 foci and relocalization to sites

of DNA-synthesis where DNA repair may occur (18). Brca1 is phosphorylated during S-phase and is also phosphorylated in response to DNA damage (18,20).

Body

In the course of identifying BRCA1-associated proteins by mass spectrometry, we identified ATM and confirmed this association by reciprocal co-immunoprecipitation. Given this physical association, we tested whether ATM was required for phosphorylation of Brca1 in response to DNA damage. We found several phosphorylation sites and showed they were ATM targets in vivo. This was all detailed in the previouys project report. Since then we have gone on to determine what other molecules might be required to help ATM phosphorylate BRCA1 since these proteins might also be tumor suppressors in the breast. We focussed on a BRCA1-related protein 53BP1. 53BP1 was originally identified through its ability to bind to the tumor suppressor protein p53 through 53BP1's C-terminal BRCT (Brca1 carboxyl terminus) repeats (21,22) which are found in many DNA damage response proteins (3-8). 53BP1 responds to DNA double strand breaks (29-32), quickly relocalizing to discrete nuclear foci upon exposure to IR. These foci colocalize with those of the Mre11/Nbs1/Rad50 complex, BRCA1 and phosphorylated γ-H2AX which are thought to facilitate recruitment of repair factors to damaged DNA (29-3). In response to IR, 53BP1 is phosphorylated in an ATM (ataxia telangiectasia mutated) dependent manner (30-32), but its role in the DNA damage response is unclear.

To determine 53BP1's role, small interfering RNAs (siRNA) in the form of two independent, non-overlapping 21-base pair RNA duplexes targeting 53BP1, were used to inhibit its expression (33). U2OS cells were transfected with these siRNA oligos and,

within three days post transfection, a portion of cells had undergone cell death (data not shown). A similar phenotype was also observed in two other cell lines, Hct116 and Saos2 (data not shown).

To determine whether 53BP1 plays a role in DNA damage cell cycle checkpoints, we examined the response of 53BP1-inhibited cells to IR. IR induces the intra-S-phase checkpoint which reduces DNA synthesis. Unlike the control cells, 53BP1-inhibited

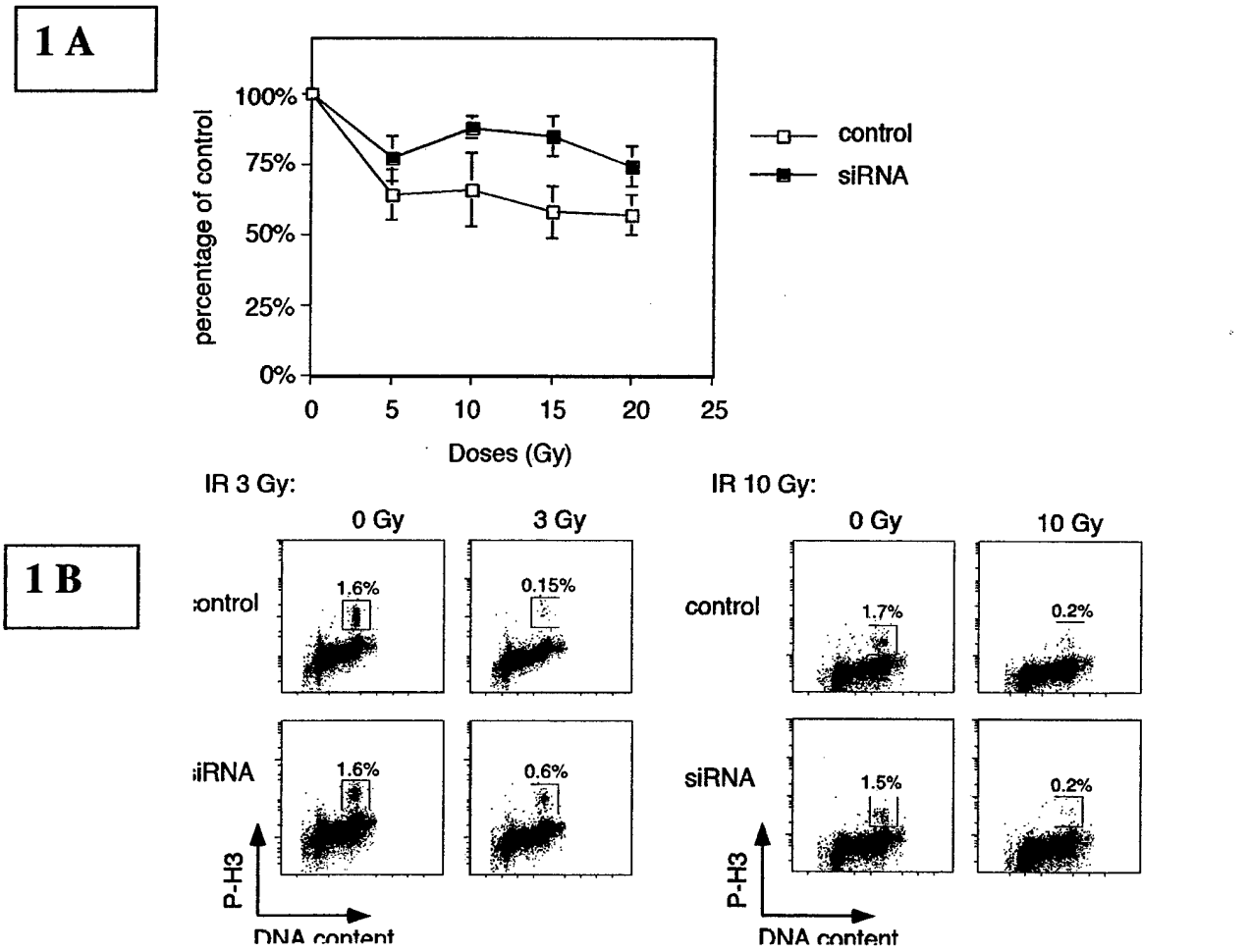

Fig. 1. 53BP1 inhibition results in defective IR-induced intra-S-phase and G2/M checkpoints. (**A**) IR-induced intra-S-phase checkpoint. Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in U2OS cells transfected with oligos. The DNA synthesis in unirradiated cultures was set to 100% for cells transfected with control oligos or siRNA oligos against 53BP1. (**B**) Analysis of the G2/M DNA damage checkpoint. Cells were either untreated or irradiated with either 3 Gy or 10 Gy as indicated, then incubated for 1 hour at 37 °C prior to fixation. Cells in mitosis were determined by staining with propidium iodide and antibody to phospho-histone H3 and percentage of the M-phase cells was determined by flow cytometry.

cells showed radio-resistant DNA synthesis (Fig. 1A). This was also seen in Saos2 and Hela cells with both siRNAs (data not shown) and indicates a role of 53BP1 in the intra-S phase checkpoint.

To assess the G2/M checkpoint, 53BP1-inhibited and control cells were irradiated with 3 or 10 Gy of ionizing radiation. Approximately three-fold more 53BP1-inhibited cells entered into mitosis than the control cells treated with 3 Gy (Fig. 1B). However, inhibition of 53BP1 had no effect following 10 Gy IR. Therefore, 53BP1-inhibited cells also displayed an IR-induced G2/M checkpoint defect. The fact that 53BP1-inhibited cells were only defective in response to lower doses of irradiation indicates the existence of an alternative signaling pathway that operates at higher doses of IR.

As 53BP1 binds p53, we asked whether 53BP1 was required for p53 activation in response to IR. P53 induction in response to IR was significantly decreased in 53BP1-inhibited cells (Fig. 2).

Fig. 2

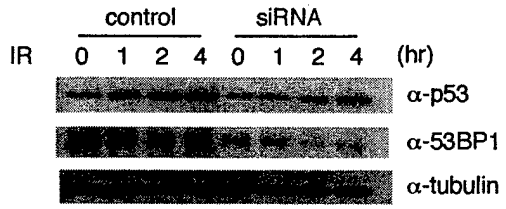

Fig. 2. 53BP1 regulates p53 in response to IR. IR-induced p53 stabilization. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then exposed to 10Gy ionizing irradiation. Cell lysates were made from samples at indicated times recovered from irradiation and separated on SDS-PAGE gel. Western blots were performed using anti-53BP1, anti-tubulin and anti-p53 antibodies.

53BP1 forms foci that overlap with BRCA1 foci in response to DNA damage. Generally there are more 53BP1 foci than BRCA1 foci and they appear to form faster than BRCA1. To test whether 53BP1 might be required for BRCA1 foci, we examined the ability of proteins to form foci in the absence of 53BP1. Brca1, Nbs1, and γ-H2AX all

form foci in response to IR (36). IR-induced Brca1 foci formation was largely abolished in 53BP1-inhibited cells. Brca1 showed diffuse staining and rarely formed distinctive foci in response to IR at different time points. In an asynchronous cell population, at 2 hr post-IR, only 4% of the cells formed Brca1 nuclear foci when cells were treated with 53BP1siRNA, compared to 60% of the control cells. Similar results were obtained in Hct116 and Hela cells with both oligo pairs. In contrast, formation of γ-H2AX foci or Nbs1 foci after IR remained unchanged in cells treated with control oligos or siRNA oligos. Rad51 foci were also unchanged.

When asynchronous control cells were analyzed for Brca1 foci formation in the absence of IR, approximately 40% contained more than 20 Brca1 foci, reflecting the S phase and G2 population. In 53BP1-inhibited cells, both the number of foci and the percentage of cells containing foci were reduced. Only 12% of 53BP1-inhibited cells contained more than 20 Brca1 foci. To control for cell cycle differences, we synchronized cells using a double-thymidine block, and S-phase cells (4 hours after release from the block) were used for immunostaining. BRCA1 foci were also dependent on 53BP1 in S-phase cells in the presence or absence of ionizing irradiation.

Although the IR-induced foci formation of Brca1 is dependent on the presence of 53BP1, Brca1 foci did not show complete colocalization with 53BP1 foci at early times. The strong effect on BRCA1 foci formation, coupled with the fact that the 53BP1 and BRCA1 foci do not initially fully overlap suggests that 53BP1 may regulate BRCA1 through a mechanism other than direct recruitment to foci. One means by which this might be achieved is through regulation of BRCA1 phosphorylation. In IR-treated cells,

Brca1 phosphorylation was reduced in the samples prepared from cells treated with siRNA oligos against 53BP1 relative to controls (Fig. 3A).

Fig. 3 A

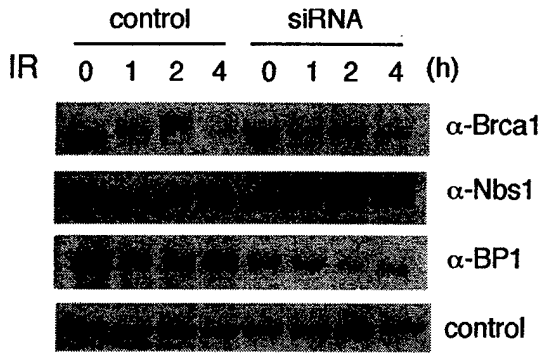

Fig. 3 B

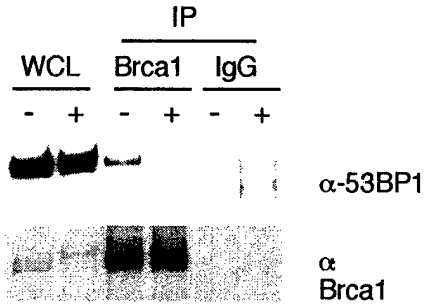

Fig. 3. 53BP1 regulation of Brca1. **(A)** Brca1 phosphorylation is reduced in the absence of 53BP1. U2OS cells were treated with siRNA oligos against 53BP1 or control oligos for two days. Cells were exposed to 10 Gy irradiation and cell lysates were prepared at indicated times after irradiation. Immunoblots were performed with antibodies against Brca1 (Oncogene), Nbs1 (Norvus) and 53BP1. The control band is a non-specific band from the same blot that was incubated with antibodies against Brca1. **(B)** 53BP1 associates with Brca1. Cell lysates from untreated U2OS cells or 2 hour after exposure to 10 Gy IR were incubated with antibodies against Brca1 or rabbit IgG as a control. Western blots were performed using anti-53BP1 and anti-Brca1 antibodies (Oncogene). Ten percent of the cell lysate used for immunoprecipitation were loaded in the control lanes (WCL).

As with the G2/M checkpoint, the strongest dependency of Brca1 phosphorylation appeared to be at lower doses of IR (not shown). High levels of IR have been shown to obscure BRCA1 regulation by other proteins such as ATM (38). Loss of 53BP1 did not have a general effect on the DNA damage-inducible phosphorylation of other proteins. Nbs1 phosphorylation was not affected (Fig. 3A). Furthermore, while BRCA1

phosphorylation showed less dependency on 53BP1 at 50Gy IR, these cells still failed to form foci (data not shown).

Next we examined whether 53BP1 associated with BRCA1. Brca1 interacts with 53BP1 in vivo, and this interaction was abolished in response to IR (Fig. 3B). Thus, this dynamic association is likely to be important for regulation of 53BP1's ability to regulate BRCA1 function in response to DNA damage.

Key research Accomplishments

A) Discovery that 53BP1 controls p53 activation

B) Discovery that 53BP1 binds to BRCA1 and releases it after DNA damage

C) Discovery that 53BP1 controls BRCA1 phosphorylation.

Reportable outcomes

None yet to report.

Conclusions

The major finding of these studies is that 53BP1 is a critical transducer of the DNA damage signal and is required for both the intra-S phase and G2/M checkpoints. It is clearly part of a partially redundant branch of the signaling apparatus and its loss results in a partial decrease in phosphorylation of key checkpoint target proteins. As it binds to p53, and Brca1 and controls BRCA1 phosphorylation, it has the property of a

mammalian adaptor or mediator that might recruit a subset of substrates to the ATM/ATR-ATRIP checkpoint kinases.

A second key finding of this study is that the pathway leading to the assembly of repair/signaling foci in response to damage is branched and shows a regulatory hierarchy in which H2AX is required for Nbs1 and 53BP1 foci (39) and 53BP1 controls the ability of at least BRCA1 but not Nbs1 to form foci. The nature of this disruption in foci formation is unknown but may be related to the role of 53BP1 in control of phosphorylation of these or other proteins. Regardless of the mechanism, it is clear that 53BP1 is a central transducer of the DNA damage signal to BRCA1 and other tumor suppressor proteins and is likely to play an important role in the maintenance of genomic stability and prevention of cancer (40,41).

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